A MURAMIDASE FROM THE FUNGUS *GLIOMASTIX MURORUM*

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ABSTRACT

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A bacterial cell wall degrading enzyme with a pH optimum of 3.4 was produced extracellularly when the fungus *Gliomastix murorum* was grown on heat-killed cells of *Bacillus subtilis* at pH5, but not at pH7. A second, much weaker, wall degrading enzyme activity with a broad pH optimum of 6-9 was produced under both growth conditions. The enzymes were not detectable in cultures grown in the absence of bacterial cells, and were apparently repressed when glucose was present in the growth medium. The low pH enzyme was purified and shown to be a muramidase with a molecular weight of about 22 000.

KEYWORDS: *Bacillus subtilis* - cell walls - degradation - *Gliomastix murorum* - muramidase - soil fungi.

INTRODUCTION

Fungi are capable of using heat-killed cells of eubacteria as well as the mycelia of other fungi and actinomycetes as sole nutrient sources (Fermor & Wood 1981, Fermor & Grant 1985). Lysis of bacterial and fungal walls occurs as part of the decomposition process, and the secretion of extracellular bacteriolytic enzymes by several fungi has recently been described (Grant *et al.* 1984, 1986). The capacity of filamentous fungi to degrade bacterial cell walls and utilise bacterial cell material as a nutrient source indicates a previously unrecognized role for fungi in the decomposition of bacterial biomass in soils. In most of the fungi studied by Grant *et al.* (1986) the bacteriolytic enzymes were either glycosidases with pH optima of 2-4, or amidases (or peptidases) with mildly alkaline pH optima. Only one fungus, *Gliomastix murorum*, produced both types of enzyme activity, depending on the culture pH. Some of the properties of the enzyme system from this fungus have been investigated in the present work.

METHODS

ORGANISMS

Gliomastix murorum, isolated from the rhizosphere of white clover growing in Manawatu pasture soil, was provided by Mr M.J. Christensen and Dr R.A. Skipp, Plant Diseases Division, Department of Scientific and Industrial Research, Palmerston North, New Zealand. The identity of this fungus has been confirmed as *G. murorum* var. *felina* by the Commonwealth Mycological Institute, Kew, Surrey, England. *Bacillus subtilis* 168 was obtained from the Microbiology Unit, Department of Biochemistry, University of Oxford, England. Cells and walls of this organism were used as fungal growth substrate and enzyme substrate respectively (Grant *et al.* 1986).

CULTURE MEDIA

G. murorum was maintained on malt extract agar (Oxoid) and was transferred to *B. subtilis* agar when required. This medium comprised heat-killed (121°C; 15 min) *B. subtilis* cells (2.5 $mg.ml^{-1}$) as sole carbon, nitrogen and phosphorus source in a mineral salts solution (Grant *et al.* 1986), buffered with 0.05 M 2-(N-morpholino)-ethanesulphonic acid (MES) (pH5) or 0.05 M N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) (pH7) and solidified with 2% (w/v) agar. For liquid cultures, agar was omitted from the *B. subtilis* medium, which was otherwise unchanged. Synthetic media had the same composition except that the heat-killed bacteria were replaced with glucose (0.1 M), po-

tassium dihydrogen phosphate (7.4 mM) and ammonium nitrate (12.5 mM) as nutrient sources.

FUNGAL CULTURES.

Starter cultures (10 ml) of *G. murorum* were grown at 25° or 30°C for 6 d. from an inoculum (0.5 cm^2) from the growing edge of an agar culture. The mycelium from starter cultures was broken into small pieces with sterile glass beads on a wrist-action shaker and used to inoculate experimental growth flasks, which were usually 250 ml Erlenmeyer flasks containing 20 or 25 ml of medium. In quantitative growth and enzyme production studies, duplicate cultures were harvested daily by centrifugation (2 800 x *g*; 15 min). Pellets were washed once with distilled water. The wash was combined with the original supernatant, adjusted to a standard volume, filtered (0.45 μm), then assayed for wall-degrading enzyme(s). Since the presence of the bacterial cells made biomass determination by direct weighing impossible, the ergosterol content of the biomass was determined by an adaptation of the method of Seitz *et al.* (1979), as follows. Mycelium was homogenized in cold methanol (25 ml) for three 30 sec periods using an Ultra Turrax homogenizer (Janke & Kunkel, W. Germany), filtered (Whatman GFC paper), then washed with methanol (2 x 5 ml). The filtrate was made up to 40 ml with methanol then saponified by refluxing for 30 min with KOH (4 g) and ethanol (10 ml). After addition of water (10 ml) to the cooled solution, ergosterol was extracted with 3 x 25 ml of redistilled hexane. The combined extracts were rotary evaporated (30-35°C) to dryness *in vacuo*, dissolved in methanol (5.0 ml) then passed through a 0.2 μm teflon filter (Gelman Acrodisc CR). Ergosterol was measured by HPLC on an Alltech C-18 5 μm column (30 x 0.46 cm), eluted with methanol containing glacial acetic acid (0.1% v/v) and water (0.5% v/v) at a flow rate of 1.8 $ml.min^{-1}$. Absorption was measured at 282 nm.

ENZYME ASSAYS

The procedures for semi-quantitative and quantitative assays of wall-degrading activity were as described previously (Grant *et al.* 1986), although the temperature used in the present work was 30°C. One unit of activity is defined as the quantity of enzyme catalysing a change in absorbance at 600 nm (ΔA_{600}) of 0.1 in 1 hr. under the standard assay conditions.

ENZYME PURIFICATION

Culture fluid was concentrated twofold by ultrafiltration at 4°C (Amicon YM-10 membrane), then ammonium sulphate (solid) was added to 70% saturation. The precipitate obtained after 16 hr. at 4°C was removed by centrifugation (18 000 x *g*; 30 min), dissolved in 0.1 M MES buffer pH 5.0, then desalted and concentrated by repeated ultrafiltration from the buffer solution. The concentrate was applied to a column (84 x 2.5 cm) of Sephadex G-75, equilibrated with 0.02 M sodium phosphate buffer, pH 7.0, previously calibrated with Blue Dextran 2000 (Pharmacia) and 1% (w/v) glucose. The column was eluted with the phosphate buffer. Enzyme-containing fractions were pooled, then desalted and concentrated by ultrafiltration. Purity of the product and molecular weight were examined by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis in an LKB Multiphor apparatus, using a 10% acrylamide gel in 0.1 M sodium phosphate buffer pH 7.2 (Hames 1981), with the following proteins as molecular weight markers: ribonuclease A (13 700), α-chymotrypsinogen A (25 700), ovalbumin (43 000) and bovine serum albumin (68 000). Gels were stained with Coomassie Brilliant Blue and de-stained with propan-2-ol/acetic acid/water (12.5:10:77.5, v/v) (Hames 1981). Molecular weight determination was also carried out by Sephadex G-75 gel filtration, using a column calibrated with the four proteins. Plots of electrophoretic migration or elution volume vs. log (M.Wt.) were linear.

ANALYTICAL METHODS

Glucose was determined by glucose oxidase (Tekit 952 DM, Searle Diagnostics Inc.) and protein by the Lowry *et al.* (1951) procedure. Reducing groups were determined as described by Park and Johnson (1949) and amino groups by the method of Ghuysen *et al.* (1966), with glucosamine and alanine, respectively, as standards. Reducing terminal residues were identified and estimated by two-dimensional paper chromatography as previously described (Grant *et al.* 1984) after sodium borohydride reduction and acid hydrolysis (Ghuysen *et al.* 1966).

RESULTS

During growth on *B. subtilis* cells buffered at pH5, *G. murorum* produced extracellular hydrolase activity against cell walls of *B. subtilis*. The pH profile of this activity showed a pronounced maximum at 3.7, with a much weaker activity between pH 5 and pH 9 (Fig. 1). In contrast, the characteristic pH 3.7 activity was not detected in culture fluids when the fungus had been grown at pH 7 under otherwise identical conditions. In these cultures, only a weak activity in the pH 6-9 range was present, similar to that in the pH 5 cultures. Although the pH 3.7 enzyme showed linear kinetics when assayed with *B. subtilis* cell walls as substrate, a non-linear progress curve was always obtained with the higher pH enzyme with reaction rate diminishing with time.

The optimum growth temperature for enzyme production was 25-30°C, and the yield of both enzymes was enhanced by agitation of the cultures and exposure to continuous light during the incubation. These results were obtained using the semi-quantitative enzyme assay on duplicate cultures grown under the various growth conditions, and were confirmed by repeating the growth experiments. Duplicate tests of several inoculation procedures showed that the highest enzyme activities were obtained when culture flasks were inoculated with *G. murorum* mycelium grown at 25°C on *B. subtilis* agar for 1 week. Under optimum conditions, enzyme activities obtained were 30-40 units ml^{-1} for the low pH enzyme (E1) and 4-5 units ml^{-1} (initial rate) for the high pH enzyme (E2).

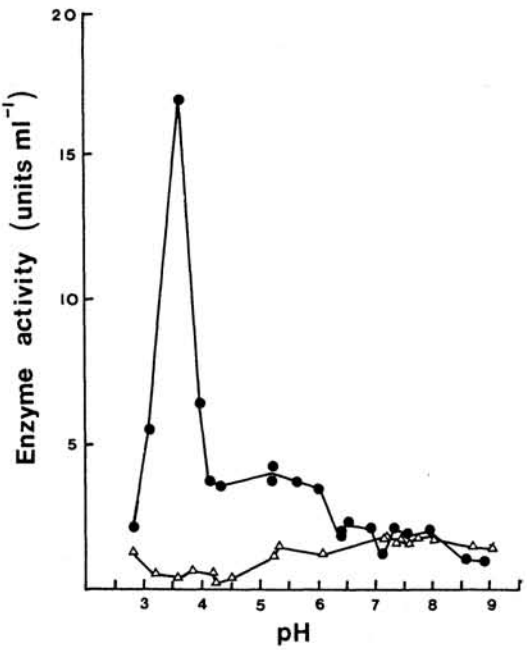

Figure 1. pH profiles of wall-degrading enzyme activity in *G. murorum* culture fluids. Activity was measured with the standard assay procedure using *B. subtilis* wall suspension in formate (pH 2.7-4.0), acetate (pH 4.4-5.8) and tris (pH 7.0-9.1) buffers, all at I=0.05. The final pH values of the reaction mixtures are shown. ●, △ fluids from culture grown at pH 5, pH 7 respectively.

Using these conditions the characteristics of production of E1 and E2 were examined during growth of *G. murorum* on *B. subtilis* liquid medium at pH 5 (E1) and pH 7 (E2). Fungal growth was expressed as ergosterol content of the total biomass (Fig. 2). Although the activity of enzyme E2 was low (Fig. 2b), it was always detectable. In contrast, no wall-hydrolysing activity was detected in culture fluids after the fungus had been grown on glucose at pH 5 or at pH 7. Addition of glucose to cultures growing on *B. subtilis* at pH 5 halted enzyme (E1) production (Fig. 3), despite increased mycelial growth.

Enzyme E1 was purified from culture fluid by ammonium sulphate precipitation followed by Sephadex G-75 gel filtration (Table 1). SDS-polyacrylamide gel electrophoresis of the final preparation showed a single protein band with an apparent molecular weight of 22 000. Molecular weight determination by gel filtration on Sephadex G-75 with protein standards gave a value of 17 000. The purified enzyme had a pH optimum of 3.4 and showed no activity above pH 3.9. It released reducing groups (Fig. 4) but no amino groups (detection limit <10% of available L-alanine residues in the peptidoglycan). The final level of reducing groups in the enzyme digest of cell walls was relatively high (0.36 μmoles.mg^{-1}) and of these reducing groups, the ratio of muramic acid to glucosamine residues was 4.5:1, as estimated by quantitative paper chromatography after acid hydrolysis of sodium borohydride-treated enzyme digests.

DISCUSSION

Growth of *G. murorum* on heat-killed cells

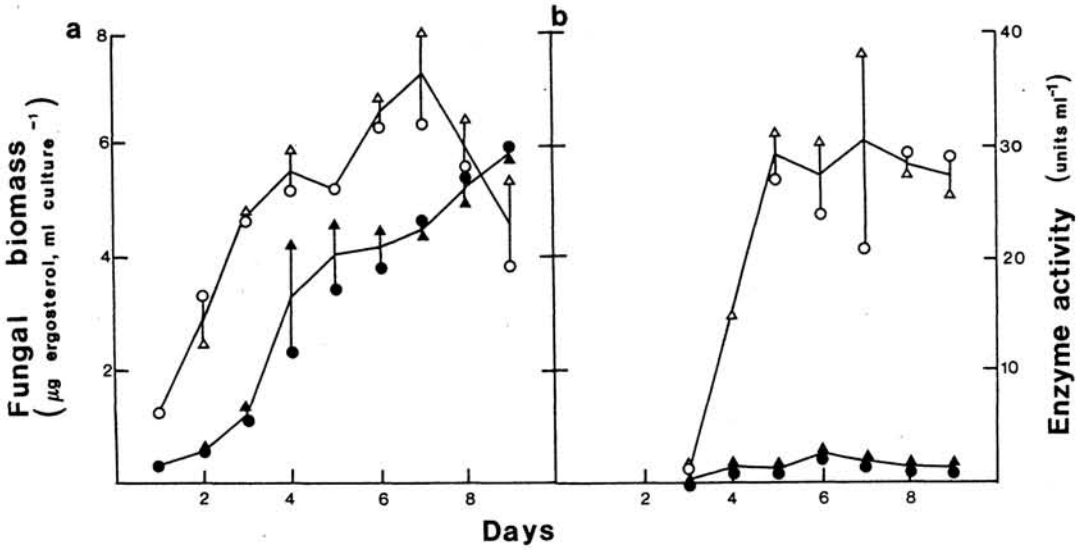

Figure 2. Growth of *G. murorum* on *B. subtilis* cells at a) pH 5 and b) pH 7; and production of extracellular wall-degrading activity. Duplicate flasks were harvested daily. Growth was measured by the ergosterol content of the biomass of individual cultures (○ , △) and enzyme activity was assayed by the standard procedure on the respective culture supernatants (●,▲) at pH 3.4 (a) and pH 7.5 (b).

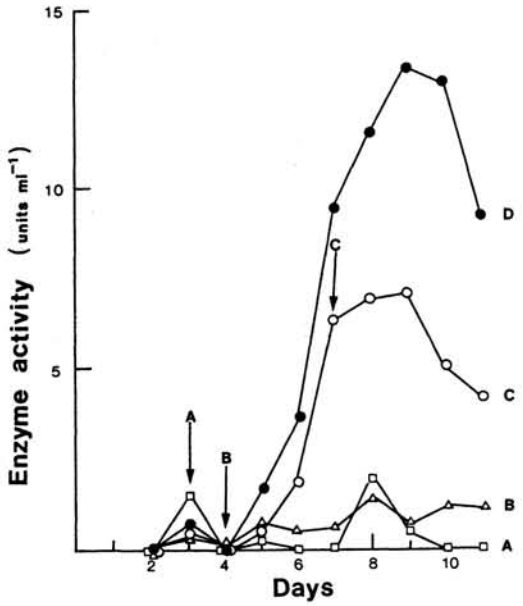

Figure 3. Effect of glucose addition on production of wall-degrading enzyme by *G. murorum* growing on *B. subtilis* cells at pH 5. Glucose (3% final concentration) was added to each of three cultures on day 3 (A,□), day 4 (B,△), and day 7 (C,○) of growth. A control culture (D, ●) had no glucose addition. Arrows show times of glucose addition to the individual cultures.

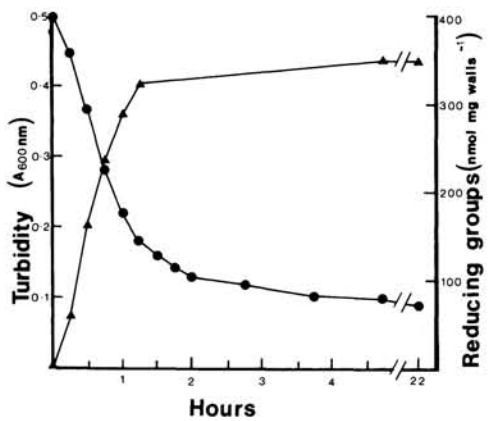

Figure 4. Time course of hydrolysis of *B. subtilis* cell walls by purified enzyme E1. ●, turbidity of cell wall suspension; ▲, reducing groups. Controls (not shown) with buffered walls or enzyme incubated separately showed no change over the incubation period.

of *B. subtilis* at pH 7 resulted in the production of a low level of extracellular cell wall degrading enzyme with a broad pH optimum of 6-9. This enzyme, which has been reported previously to release amino groups during its attack on bacterial walls (Grant *et al*. 1986), showed non-linear

Table 1. Purification of Enzyme E1 from culture fluid of *G. murorum* grown on *B. subtilis* cells at pH5.

Step	Protein (mg)	Total Activity (units)	Specific Activity (units.mg^{-1})	Yield (%)	Purification (fold)
1. Crude supernatant	11.5	3.07	0.27	100	1
2. $(NH_4)_2SO_4$ precipitate	0.55	1.82	3.31	59.2	12.3
3. Sephadex G-75	0.02	0.80	40.0	26.1	148.1

kinetics and was not produced when the fungus was grown in the absence of bacteria. However, when *G. murorum* was grown under otherwise identical conditions, but at pH 5 rather than pH 7, it produced, in addition to this relatively weak activity, a highly active wall-degrading enzyme with a sharp pH optimum of 3.4. This activity was never detected during growth at the higher pH.

The pH 3.4 enzyme, E1, which was purified by a simple, two-step procedure, was found to be a muramidase, although the results indicated that it may have possessed some glucosaminidase activity as well. The proportion of reducing groups released by the enzyme from B. subtilis cell walls was relatively high (0.36 μmoles.mg^{-1}) considering that the approximate combined content of muramic acid and glucoasamine residues in *B. subtilis* 168 walls is only 0.6 μmoles.mg^{-1} (Hughes *et al.* 1968). Similar, though slightly lower, results were obtained earlier with the crude *G. murorum* culture fluid (Grant *et al.* 1986). It seems probable that production of enzyme E1 was induced by bacterial cells or cell components, since growth of the fungus in the absence of bacteria failed to produce enzyme activity. Synthesis of this enzyme was also repressed by glucose (Fig. 3) and although detailed studies need to be made of the production of the high pH enzyme, E2, it is interesting to note that it was absent also in glucose-grown cultures. Sensitivity to catabolite repression was indicated by the higher enzyme activities obtained when the fungal inocula were taken from growth on *B. subtilis* agar as compared with glucose-based agar. It was shown recently that the acidic muramidase from *Schizophyllum commune* was inducible by bacterial cells or cell components and repressed by glucose (W.D. Grant, B.A. Prosser & R.A. Asher, unpublished results). Although the control of bacteriolytic enzyme production has not yet been fully investigated in any microbial system, Hash (1974) claimed that the lysozyme from *Chalaropsis* was constitutive: addition of bacterial cell walls to the fungal growth medium had no effect on production of the enzyme. Whether such a system can truly be called "constitutive" is open to question since the enzyme was produced only in certain growth media and not in others (Hash & Rothlauf 1967). Ward & Perkins (1968) also found that bacterial cells or walls did not stimulate bacteriolysin production by *Streptomyces griseus*. However, in contrast, such stimulation has been observed in several other strains of *Streptomyces* (Yokogawa *et al.* 1973, Hayashi *et al.* 1981).

The molecular weight of the enzyme obtained by Sephadex G-75 gel filtration was 17 000, whereas SDS-polyacrylamide gel electrophoresis gave a value of 22 000. Although analytical centrifuge measurements would be necessary to establish which of the two results was correct, it seems likely that the electrophoresis value would be more reliable: adsorption of lysozyme to Sephadex gels has been reported previously (Whittaker 1963, Ward & Perkins 1968), and this could result in a lower apparent molecular weight. It is of interest that *Chalaropsis* muramidase had a molecular weight of 23 385, as calculated from its amino acid composition (Shih & Hash 1971), and ultracentrifuge studies yielded a value of 21 000 for this enzyme (Mitchell & Hash 1969).

The most striking aspect of the bacteriolytic system of *G. murorum* was the production of a major extracellular enzyme activity during growth at low pH, and the complete absence of this activity when the fungus was grown at pH 7; the mechanism of regulation of this enzyme would provide an interesting study. One possible reason for such an unusual feature is that at higher pH values fungi are more capable of attacking heat-killed bacteria by cytolysis, i.e., by degrading cytoplasmic contents without damag-

ing the cell wall (Grant *et al.* 1986). Bacteriolysins are thus not needed for bacterial decomposition at alkaline pH. We have shown that alkaline proteases secreted by the fungi are responsible, at least in part, for this process (Grant, Prosser & Wakefield, unpublished work).

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